Geoderma 316 (2018) 1–10

Contents lists available at ScienceDirect

Geoderma

journal homepage: www.elsevier.com/locate/geoderma

Long-term warming rather than grazing significantly changed total and active soil procaryotic community structures

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ARTICLE INFO

Keywords:
Climate changes
Livestock grazing
Tibetan plateau
Soil microbes
Microbial activity

ABSTRACT

There is a paucity of knowledge in understanding the effects of warming and grazing on soil microbes and their active counterparts, especially on the Tibetan Plateau which is extremely sensitive to global warming and human activities. A six-year field experiment was conducted to investigate the effects of asymmetric warming and moderate grazing on total and active soil microbes in a Tibetan *Kobresia* alpine meadow. Soil bacterial abundance and 16S rDNA transcriptional activity were determined using real-time PCR. Total and active soil procaryotic community structures were analyzed through MiSeq sequencing based on 16S rDNA and rRNA, respectively. The results showed that the soil procaryotic community was more sensitive to the warming than the grazing. The warming significantly decreased soil microbial respiration rates, 16S rDNA transcription activity, and dispersion of total procaryotic community structures, but significantly increased the α diversity of active procaryotes. Warming also significantly increased the relative abundance of oligotrophic microbes, whereas decreasing the copiotrophic lineage proportions. The functional profiles predicted from the total procaryotic community structures remained unaffected by warming. However, the rRNA-based predictions suggested that DNA replication, gene expression, signal transduction, and protein degradation were significantly suppressed under the warming. The grazing only significantly decreased the 16S rDNA transcription and total procaryotic richness. Overall, these findings suggest that warming can shift soil procaryotic community to a more oligotrophic and less active status, highlighting the importance of investigating active microbes to improve our understanding of ecosystem feedbacks to climate change and human activities.

1. Introduction

The unequivocal global warming profoundly alters terrestrial biodiversity and ecosystem functioning, which, in turn, can influence the ongoing global climate changes (IPCC, 2013). Accordingly, soil microbes have been intensively examined in temperature manipulation investigations by virtue of their vital roles in determining ecosystem feedbacks (Melillo et al., 2017; Oliverio et al., 2017; Romero-Olivares et al., 2017). Furthermore, the interactive effects between warming and human activities (e.g., grazing) on soil microbes have also been observed in a number of studies (Li et al., 2016; Zhang et al., 2016b). However, most of these studies only focused on the responses of total soil microbial communities (Knox et al., 2017; Zhang et al., 2017; Zhang et al., 2016b). The active microbes have rarely been investigated due to the difficulties in soil labeling and RNA extractions (Che et al., 2016a). Nevertheless, soil microbes have a wide range of vitality, with a small active population being active, while the majority is dormant (Fierer, 2017; Lennon and Jones, 2011). Compared to total soil microbes, active soil microbes usually show higher sensitivity to environmental changes (Barnard et al., 2015; Che et al., 2015; Xue et al., 2016b), and are more connected with soil functionality (Che et al., 2016b). Therefore, examining the effects of warming and human activities on active soil microbial communities can improve our understanding of ecosystem feedbacks to the future climate changes.

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https://doi.org/10.1016/j.geoderma.2017.12.005
Received 1 September 2017; Received in revised form 5 December 2017; Accepted 6 December 2017
Available online 18 December 2017

In the recent decade, rRNA-based methods (e.g., rRNA sequencing) have been widely employed to identify active soil microbial communities (Barnard et al., 2015; Che et al., 2015; Che et al., 2016b; Mueller et al., 2016) for the following reasons. First, rRNA genes (rDNAs) are the most widely-used molecular markers to determine microbial abundances and community compositions (Che et al., 2016b). Second, rRNAs are indispensable for protein synthesis, and in some pure cultures, their cellular concentration correlate well with microbial growth rates (Kerkhof and Kemp, 1999; Muttray and Mohn, 1999; Perez-Osorio et al., 2010). Third, the dormancy and decease of microbes usually accompany rRNA degradation (Lahtinen et al., 2008; Segev et al., 2012). Fourth, the rRNA-based methods can identify active microbes without labeling, which avoids disturbance and captures more reliable active microbial profiles. Moreover, the invention and popularization of the commercial soil RNA extraction kits have largely solved the difficulties in soil RNA extraction (e.g., Che et al., 2017). Consequently, a combination of the rDNA- and rRNA-based methods can systematically determine the responses of both total and active soil microbes (Che et al., 2016a; Che et al., 2016b).

Tibetan Plateau, known as the third pole of our planet, is extremely sensitive to global warming (Chen et al., 2013; Chen et al., 2015; Liu and Chen, 2000) and human activities, particularly livestock grazing (Chen et al., 2013; Zhou et al., 2005). There are 23.2 Pg of organic carbon (2.5% of the global pool) stored in the Tibetan soils (Wang et al., 2002), and thus even a slight variation in this organic carbon pool can result in a considerable feedback to the global warming. Soil microbes, especially the active populations, play a key role in determining the dynamics of soil organic carbon pools (Che et al., 2016b). Moreover, they have been also recognized as a vital factor for influencing the sustainability of Tibetan alpine meadows (Che et al., 2017). Therefore, determining the individual and combined effects of warming and grazing on soil microbial community is not only important for predicting the Tibetan ecosystem feedbacks to global warming, but also crucial to providing a basis for optimizing the grazing strategies under the climate change.

In Tibetan alpine meadows, the previous studies suggested that warming significantly increased soil respiration, litter mass losses, phosphomonoesterase activity, soil nutrient contents, and belowground biomass, and significantly affected soil N_2O emission and plant community composition (Hu et al., 2010; Lin et al., 2011; Luo et al., 2010; Luo et al., 2009; Rui et al., 2011; Rui et al., 2012; Wang et al., 2012). Compared to warming, the effects of grazing were generally weak and sometimes converse (Jiang et al., 2016; Wang et al., 2012). The most significant effect of grazing was the decrease in litter input (Luo et al., 2009). In addition, significant warming-grazing interactive effects were observed in several studies (Li et al., 2016; Rui et al., 2011; Wang et al., 2012). Responses of soil microbes to warming and grazing were analyzed in a few investigations (Li et al., 2016; Yang et al., 2013; Zhang et al., 2016a; Zhang et al., 2016b). However, as mentioned above, these studies only assessed the total soil microbes, while the effects of warming and grazing on the active soil microbes have never been investigated in this region. In addition, although soil microbes should be sensitive or responsive to the aforementioned changes, significant effects of warming and grazing on total soil bacterial communities were seldom observed in the previous studies (Li et al., 2016; Zhang et al., 2016b).

Therefore, in this study, we aimed to investigate the main and interactive effects of warming and grazing on total and active soil prokaryotes in a *Kobresia* alpine meadow on the Tibetan Plateau. Bacterial abundance and 16S rRNA transcriptional activity were determined using 16S rDNA and rRNA copies, respectively. Total and active procaryotic community structures were analyzed using Miseq sequencing based on 16S rDNA and rRNA, respectively. On the basis of the aforementioned findings, we hypothesized that: 1) warming and grazing exerted more profound effects on active prokaryotes than total prokaryotes in soils; 2) warming stimulated soil microbial activity and 16S rDNA transcription; and 3) soil prokaryotes were less sensitive to grazing than warming, but grazing could partially offset the effects of warming.

2. Materials and methods

2.1. Study site

This research was conducted at the Haibei Alpine Meadow Ecosystem Research Station (37° 37′ N, 101° 12′ E), situated in the northeast of the Tibetan Plateau. With an average elevation of 3200 m, this region has a typical plateau continental climate. The mean annual temperature and precipitation were − 1.7 °C and 570 mm, respectively (Zhao et al., 2006). The soil was classified as Gelic Cambisols (WRB, 1998). The vegetation of our study site was dominated by species such as *Potentilla nivea*, *Kobresia humilis*, and *Elymus nutans* (Wang et al., 2012). The average coverages of graminoids, forbs, and legumes were about 86%, 86%, and 28%, respectively (Wang et al., 2012). A detailed description of the experimental site can be found in our previous studies (Jiang et al., 2016; Ma et al., 2015).

2.2. Experimental design

The field treatments first started in May 2006 and continued until 2012 to reveal the effects of warming and grazing on alpine meadow ecosystems. The experimental design has been provided in our previous studies (Jiang et al., 2016; Luo et al., 2009). It was a two-way factorial design with four replicates. In total, 16 orbicular plots (3 m diameter) were established in the field with a randomized complete block design. The interval between every two adjacent plots was 3 m.

The experimental warming was achieved by an infrared heating system named free-air temperature enhancement (FATE) that has been detailed by Luo et al. (2009). The canopy temperature increase in the warming plots was 1.2 °C during the day and 1.7 °C at night in the growth seasons (May to September), while it was 1.5 °C in the daytime and 2.0 °C at night in the non-growth seasons (October to April). These temperature increases were in accordance with the predictions of global warming (IPCC, 2013). In each plot, type-K thermocouples (Campbell Scientific, Logan, Utah, USA) were used to automatically measure soil temperature at 0–5 and 5–10 cm. All temperature data were recorded and stored in CR1000 dataloggers.

From August 2006 to October 2010, the grazing plots were grazed by Tibetan domestic sheep (*Ovis aries*; Table 1). One or two sheep were fenced in the plots for 1 to 2 h to remove plant to approximately half of the canopy height. The details of the sheep grazing treatments have been described in our previous study (Rui et al., 2012). In November 2011 and April 2012, instead of sheep grazing, clipping was employed to simulate winter grazing (Table 1). The clipping removed approximately 90% of the grass litter. Overall, these grazing treatments were

Table 1
Methods of grazing treatments.

Time	Methods	Intensity[a]
August 2006	Sheep grazing	50%
July 2007	Sheep grazing	50%
August 2007	Sheep grazing	50%
September 2007	Sheep grazing	50%
July 2008	Sheep grazing	50%
August 2008	Sheep grazing	50%
July 2009	Sheep grazing	50%
August 2009	Sheep grazing	50%
July 2010	Sheep grazing	50%
August 2010	Sheep grazing	50%
November 2011	Clipping	90%
April 2012	Clipping	90%

[a] The grazing intensity was indicated by the variations of canopy height.

approximately equivalent to local moderate grazing.

2.3. Soil sampling and the measurements of soil properties and belowground biomass

The soil samples (0–10 cm depth) of each plot were collected, using a steel auger, from five points, in August 2012. After obvious roots were manually removed from the soils, the soil samples for nucleic acid extraction were immediately flash-frozen and stored in liquid nitrogen. The soils were preserved at − 80 °C in the laboratory. The soil samples used for soil physicochemical property analysis were first sieved to ≤ 2 mm, then transported to the laboratory in an insulated box with ice blocks, and then preserved at − 20 °C. In addition, a soil core of 20-cm diameter in each plot was sampled to determine the belowground plant biomass.

Soil gravimetric moisture was measured by drying fresh soils at 105 °C for 24 h. Soil dissolved organic carbon (DOC), NH_4^+-N, NO_3^--N contents were analyzed using the 2 M KCl extraction (soil mass to extractant ratio of 1:4). After the removal of carbonate by adding HCl, the extracted DOC concentrations were determined using a TOC Analyzer (Liqui TOC II; Elementar Analysensysteme GmbH, Hanau, Germany). Then, the concentrations of soil NH_4^+-N, NO_3^--N were measured using an auto-flow analyzer (Auto Analyzer 3 System; SEAL Analytical GmbH, Norderstedt, Germany). Soil total carbon and nitrogen contents were analyzed with an automatic elemental analyzer. Soil pH was determined using a pH meter in a 1:5 soil-water suspension. The soil microbial biomass was measured with the chloroform fumigation-extraction method, as detailed in our previous report (Ma et al., 2015).

Soil microbial respiration was measured as the CO_2 production rates using the soils sieved to ≤ 2 mm. Field-moist soil samples, containing 10 g of dry mass, were placed into 130-ml flasks which were then pre-incubated at 20 °C, with good aeration, for seven days to stabilize the soil conditions. On the eighth day of the incubation, the flasks were sealed with rubber stoppers. After 1 h, 10 mL of the headspace air of each flask was sampled using a 30-mL syringe, every 2 h, for four times. The CO_2 concentrations were determined using an Agilent 7890A gas chromatograph. The soil microbial respiration rates were calculated based on the increase of CO_2 concentrations over time.

2.4. Nucleic acid extraction and cDNA synthesis

We used 0.3 and 2.0 g of soil to extract DNA and RNA, respectively. The extractions were conducted separately using a PowerSoil™ DNA Isolation Kit and a PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The RNA extracts were treated with DNase I (MO BIO Laboratories, Carlsbad, CA, U.S.A.) to remove DNA residuals. After that, the RNAs were reverse-transcribed into cDNA using a PrimeScript™II 1st Strand cDNA Synthesis Kit with random hexamers (Takara Bio Inc., Shiga, Japan). The DNA and cDNA solutions were diluted 10 and 50 times respectively for the subsequent operations.

2.5. Real-time PCR

The copy numbers of 16S rDNA and its transcript were determined using an ABI StepOne Plus® Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) with the universal bacterial primer set (338F, 5′-ACT CCT ACG GGA GGC AG-3′, Lane, 1991; 518R, 5′-ATT ACC GCG GCT GCT GG-3′, Muyzer et al., 1993). Each of the 25 μL reaction mixture contained: 1 μL template DNA (DNA, cDNA, or 10-fold serially diluted standards), 12.5 μL Maxima™ SYBR Green/ROX (2 ×, Fermentas, Waltham, MA, USA), 0.5 μL forward primer (20 μmol L^{-1}), 0.5 μL reverse primer (20 μmol L^{-1}), 0.25 μL bovine serum albumin (25 mg mL^{-1}; Promega, Madison, WI, USA), and 10.25 μL nuclease-free water. Standard curves were constructed using plasmids harboring 16S rDNA fragments. All the DNAs and cDNAs were analyzed in triplicate, and three no template controls were used to check the contamination of reagents. The PCR runs started with an initial denaturation and enzyme activation step for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C, and 15 s at 80 °C. We recorded fluorescence signal at 80 °C to attenuate influence of primer dimers. The specificity of PCR products was tested by melting curve analysis. The amplification efficiencies were around 95%; the r^2 values of the standard curve were > 0.995. The absence of PCR inhibitors in RNA, DNA, and cDNA solutions was checked via qPCR with dilution series of corresponding solutions (Hargreaves et al., 2013).

2.6. MiSeq sequencing and bioinformatics

The PCR amplicons for the MiSeq sequencing were generated with barcoded universal primers for prokaryotes (515F, 5′-GTG CCA GCM GCC GCG GT AA-3′, Caporaso et al., 2011; 909R, 5′-CCC CGY CAA TTC MTT TRA GT-3′, Wang and Qian, 2009). The 12 bp barcode sequences were added to the 5′-end of 515F. The 50 μL reaction system contained: 1 μL template DNA, 25 μL Premix Taq™ Hot Start Version (Takara Bio Inc., Shiga, Japan), 1 μL each primer (20 μmol L^{-1}), and 22 μL water. The PCR programs were as follows: initial denaturation at 94 °C for 1 min, followed by 30 cycles of 20 s at 95 °C, 30 s at 56 °C, and 45 s at 72 °C, ending with a final extension at 72 °C for 10 min. There were two technical replicates for each sample, and the PCR products from two technical replicates were pooled into one tube. The PCR products were then purified using the GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania). Subsequently, all the samples were pooled together with an equal molar amount from each sample.

The MiSeq sequencing was conducted by the analytical center at the Chengdu Institute of Biology, Chinese Academy of Sciences. The sequencing samples were prepared using a TruSeq DNA kit following the manufacturer's instructions. The purified products were diluted, denatured, re-diluted, and mixed with PhiX (equal to 30% of final DNA amount) as described in the Illumina library preparation protocols, and were then applied to an Illumina Miseq system for sequencing with the Reagent Kit v3 2 × 300 bp.

The sequence data were processed using a series of tools including “Quantitative Insights Into Microbial Ecology” (Qiime; v1.9.1; Caporaso et al., 2010), Mothur (v1.27; Schloss et al., 2009), Usearch (v8.0.1623; Edgar, 2010), and R (R Development Core Team, 2016). The paired-end sequences were first spliced and assigned to each sample based on their barcodes using Qiime. Then, the contigs were analyzed using the default UPARSE operational taxonomic unit (OTU) analysis pipeline (Edgar, 2013) to generate an OTU table and the OTU representative sequences. Specifically, the OTUs were clustered at a 97% identity threshold. The taxonomic identities of the OTU representative sequences were determined against the Silva database (v128) with pseudo-bootstrap confidence score = 80% (Wang et al., 2007). Finally, all the samples were rarefied to 15,955 sequences to calculate α diversity, β diversity, and the composition of each sample using R with vegan packages (Oksanen et al., 2017). The prokaryotic community dispersion was calculated using *betadisper* functions in vegan package, and presented as the distance to centroid based on PCoA ordination. In addition, we calculated the relative abundance of copiotrophic and oligotrophic lineages based on their responses to organic matter amendments in the published investigations (Che et al., 2016b; Ho et al., 2017). Specifically, the copiotrophic lineages included Bacteroidetes, Betaproteobacteria, and Gammaproteobacteria. The oligotrophic lineages included Archaea, Acidobacteria, Actinobacteria, Planctomycetes, Verrucomicrobia, and Chloroflexi. All the raw sequencing data have been deposited in the NCBI Sequence Read Archive under the BioProject PRJNA417160, with accession number from SAMN07977869 to SAMN07977900.

The microbial functional profiles were predicted using the “phylogenetic investigation of communities by reconstruction of unobserved states” (PICRUSt; Langille et al., 2013). PICRUSt is an approach using

an extended ancestral-state reconstruction algorithm to predict microbial functional compositions on the basis of 16S rRNA gene profiles (Langille et al., 2013). In this study, the closed reference OTU table for PICRUSt was generated using QIIME following the online instructions with the UCLAST algorithm and Green Gene reference database. The PICRUSt analysis was performed using the online Galaxy version of the Langille Lab (galaxy.morganlangille.com). The function predictions were performed based on KEGG orthology at level three.

2.7. Statistical analysis

The main and interactive effects of warming and grazing on the soil properties, plant biomass, bacterial abundance, 16S rDNA transcriptional activity, microbial respiration rates, and procaryotic diversities were analyzed using the two-way analysis of variance (ANOVA) followed by the Duncan's test. The normality and homogeneity of variance were tested by Shapiro and Bartlett test, respectively. We performed non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) to reveal the effects of warming and grazing on the total and active soil procaryotic community and predictive function profiles. All the community composition distances were calculated based on Bray-Curtis dissimilarities. The responses of the relative abundance of procaryotic lineages (from phylum to genus) and the predictive functions to warming were further determined using the "linear discriminant analysis effect size" (LEfSe) method (Segata et al., 2011). The Pearson correlation test, envfit based on NMDS, and multivariate regression tree analysis were applied to determine the relationships between environmental factors and soil procaryotic communities. The LEfSe analysis was performed using the online Huttenhower Galaxy server (huttenhower.sph.harvard.edu/galaxy) with default pipelines. All the other statistical analyses were conducted using R with vegan and mvpart packages.

3. Results

3.1. Soil and plant properties

Both the warming and grazing significantly increased the soil temperature ($P < 0.001$, Fig. S1a). On average, the warming and grazing caused a 1.57 °C and 0.98 °C rise in soil temperature, respectively (Fig. 1a). However, no significant effects of interaction between the warming and grazing on soil temperature were observed (Table S1). Warming significantly reduced the soil gravimetric moisture by 11.91% ($P < 0.001$, Fig. S1b), while no significant effects of grazing and warming-grazing interaction on soil gravimetric moisture were observed (Table S1, Fig. S1b).

In addition, the warming significantly decreased the soil NH_4^+-N contents ($P = 0.022$; Fig. S1d) and increased the belowground plant biomass ($P = 0.042$; Fig. S1f). The soil DOC contents tended to increase under warming, although the increase was not statistically significant ($P = 0.069$; Fig. S1e). The warming-grazing interactions significantly offset the individual effects of warming and grazing on the soil NH_4^+-N contents ($P = 0.016$; Fig. S1d) and pH values ($P = 0.002$; Fig. S1c). All the other soil properties included in this study remained unaffected under the treatments (Table S1).

3.2. The soil bacterial abundance, 16S rDNA transcriptional activity, and microbial respiration rates

As shown in Fig. 1, warming significantly decreased the 16S rRNA copies ($P = 0.029$), the rRNA-rDNA ratios ($P = 0.015$), and the microbial respiration rates ($P = 0.008$). The grazing significantly decreased the 16S rRNA copies ($P = 0.045$) and rRNA-rDNA ratios ($P = 0.041$; Fig. 1). There were no significant interactive effects between the warming and grazing on any of these indices (Table S2; Fig.1), and no significant responses of soil bacterial abundance to these treatments were observed (Table S2). The soil microbial respiration rates showed significant correlations with the 16S rRNA copies ($r = 0.530$, $P = 0.035$) and rRNA-rDNA ratios ($r = 0.584$, $P = 0.018$) rather than the 16S rDNA copies ($r = -0.196$, $P = 0.467$).

3.3. The total and active procaryotic α diversities

In total, we obtained 3609 OTUs for all the sequences. The average richness coverages for the 16S rDNA and rRNA sequencing were 71.0% and 73.9%, respectively. The total soil procaryotic α diversities remained unaffected by the warming treatments (Table S2; Fig. 2a, b, and c), but the total soil procaryotic richness significantly reduced under the grazing ($P = 0.042$; Fig. 2b). On the contrary, the active soil procaryotic α diversities were highly sensitive to the warming but not grazing. Specifically, the active soil procaryotic Shannon diversity ($P < 0.001$), richness ($P < 0.001$), and evenness ($P < 0.001$) were all significantly increased under the warming (Table S2, Fig. 2e, f, and g).

3.4. The total and active soil procaryotic community structures

As shown in Fig. 3, the dominant phylum in both the total and active procaryotic communities was Proteobacteria (40.78%), followed by Acidobacteria (20.43%), Bacteriodetes (15.23%), Planctomycetes (7.01%), Actinobacteria (4.37%), and Planctomycetes (2.85%). The relative abundance of archaea was extremely low in the total (1.33%) and active (0.75%) procaryotic communities, and 96.15% of the archaea were classified as Thaumarchaeota which were further identified as the soil Crenarchaeotic group.

The most abundant families in the total and active procaryotic communities were Chitinophagaceae (10.03%) and Comamonadaceae (9.11%). The proportions of Planctomycetaceae, Cytophagaceae, Blastocatellaceae (Subgroup 4), Nitrosomonadaceae, and

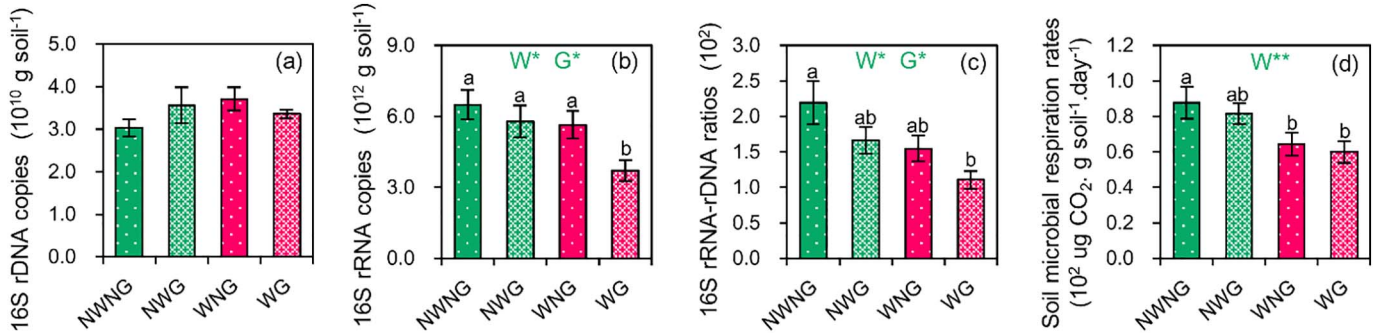

Fig. 1. The soil 16S rDNA copies (a), 16S rRNA copies (b), 16S rRNA-rDNA ratios (c), and microbial respiration rates under different treatments. NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; WG: warming with grazing; W: effect of warming; G: effect of grazing. All the data were presented in mean ± SE, $n = 4$. Bars with different letters indicate significant differences at $P < 0.05$.

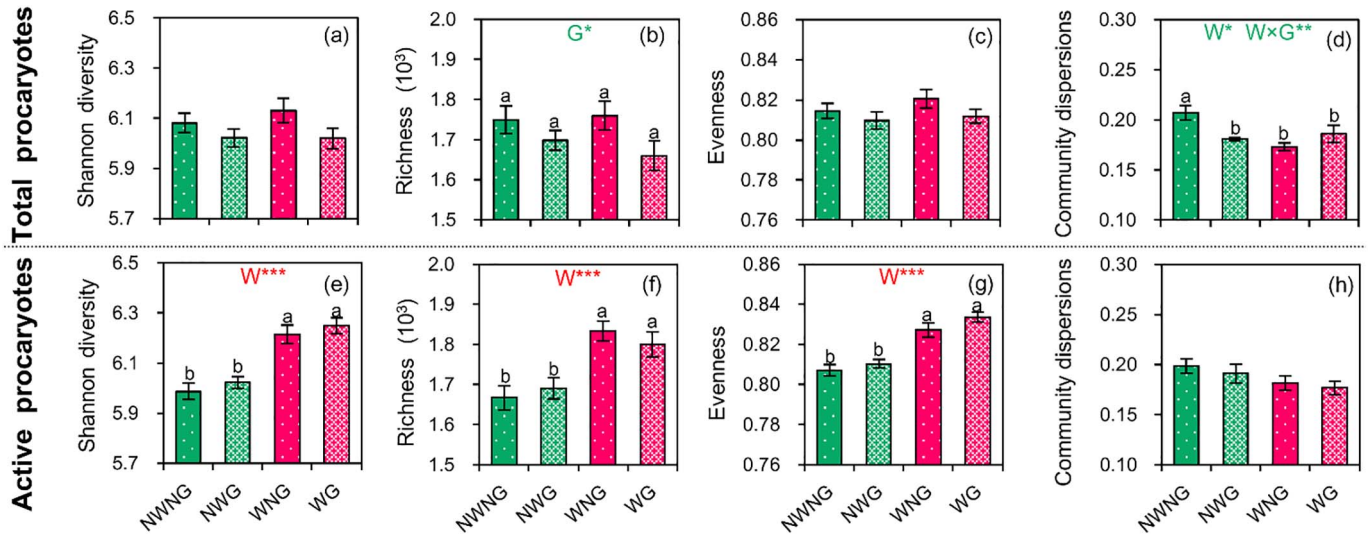

Fig. 2. The total and active soil procaryotic diversity indices under different treatments. The prokaryotic community dispersion was represented as the distance to centroid based on PCoA ordination. NWNG: no-warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; WG: warming with grazing; W: effect of warming; G: effect of grazing; W × G: interaction effect of warming and grazing. All the data were presented in mean ± SE, $n = 4$. Bars with different letters indicate significant differences.

Xanthomonadales (Incertae_Sedis) were also high in both the total and active procaryotic communities (Fig. S2). The Chitinophagaceae were mainly classified as *Terrimonas*, *Ferruginibacter*, and *Niastella*, while most of the Comamonadaceae were identified as *Piscinibacter* and *Caenimonas*. In addition, RB41, *Haliangium*, *Bradyrhizobium*, and *Acidibacter* were also identified as abundant genera in the procaryotic communities (Fig. S3). The community compositions at family and genus levels were detailed in Figs. S2 and S3.

The NMDS and PERMANOVA suggested that the total and active procaryotic communities responded similarly to the treatments, although they differed significantly from each other ($P < 0.001$). Specifically, the warming significantly altered both the total ($P = 0.006$) and active ($P = 0.002$) procaryotic community structures (Table S2, Fig. 4a and b). Conversely, neither the grazing nor the warming-grazing interaction could significantly alter the procaryotic community structures (Table S2, Fig. 4a and b).

In addition, the dispersion of total procaryotic communities in the control plots were significantly higher than those in the other plots (Figs. 2d and 4a). However, the warming-grazing interaction significantly offset the individual effects of warming and grazing (Figs. 2d and 4a). Conversely, the dispersion of the active procaryotes remained unaffected across all the treatments (Table S2; Figs. 2h and 4b).

As revealed by the LEfSe analysis, the relative abundance of β-Proteobacteria in the total and active procaryotic communities significantly decreased under the warming (Fig. 5a and b). Conversely, the relative abundance of Actinobacteria significantly increased (Fig. 5a and b). In addition, as for the active procaryotic communities, the warming significantly increased the relative abundances of Firmicutes, Chloroflexi, α-Proteobacteria, and some less abundant lineages under δ-Proteobacteria, but decreased the proportions of Verrucomicrobia, Myxococcales, and Solibacteres (Fig. 5b).

The proportions of copiotrophic lineages in the total and active soil procaryotic communities significantly decreased under the warming (Fig. 6a and c). On the contrary, the warming significantly increased the relative abundance of oligotrophic lineages (Fig. 6b and d). The copiotroph proportions were positively correlated with soil microbial respiration rates, while they were negatively correlated with the belowground plant biomass (Figs. S4a, S4c, S5a, and S5c). In contrast, the proportions of oligotrophs showed negative and positive correlations with the soil microbial respiration rates and the belowground plant biomass, respectively (Figs. S4b, S4d, S5b, and S5d).

The envfit analysis suggested that the total soil procaryotic community structures had significant correlations with the soil temperature and moisture, while the active procaryotic community structures were significantly correlated with the soil temperature, moisture, NO_3^--N content, inorganic nitrogen content, and the plant belowground biomass (Fig. S6; Table S3). In addition, the soil microbial respiration rates also showed significant correlations with the total ($r^2 = 0.7220$,

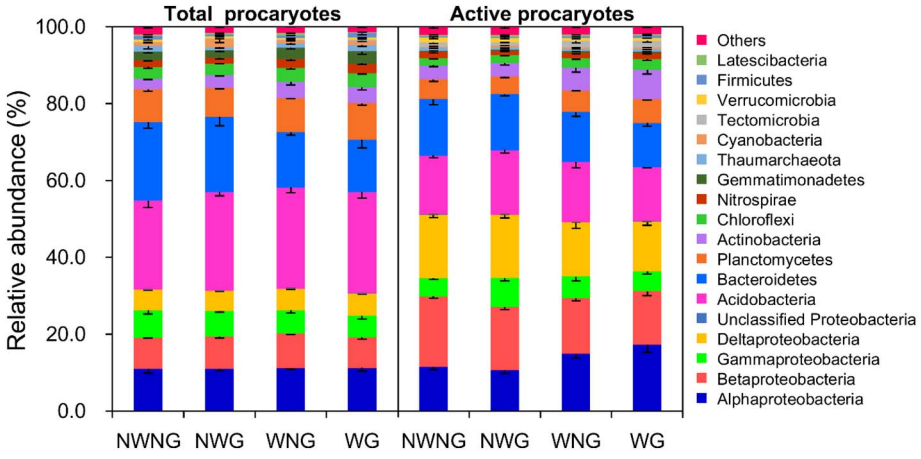

Fig. 3. The total and active soil procaryotic community compositions under different treatments. NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; WG: warming with grazing. Others: the sum of phylum occupying < 0.5% of the total population. All the data were presented in mean - SE, $n = 4$.

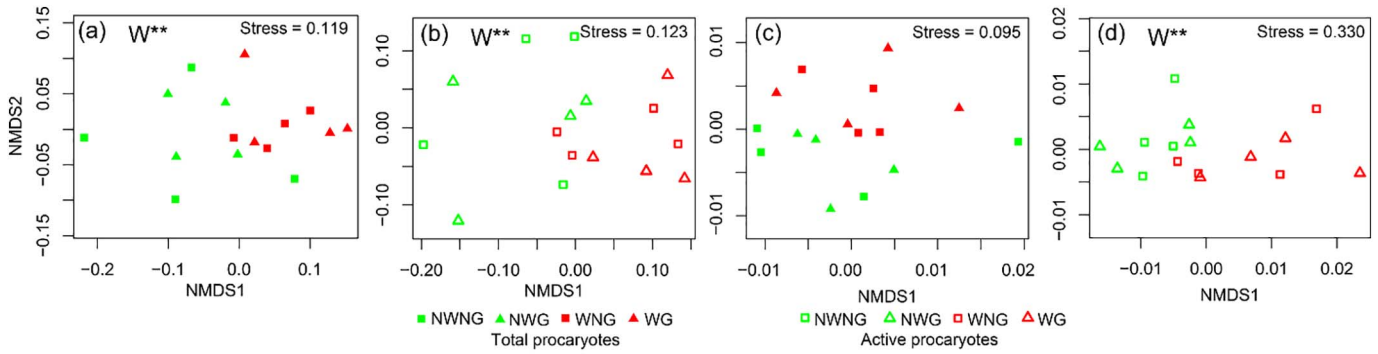

Fig. 4. The NMDS ordinations of the total and active soil procaryotic community structures based on OTUs (a and b) and putative functions (c and b). NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; WG: warming with grazing. The putative functions were determined through PICRUSt analysis with KEGG pathway assignment at level three.

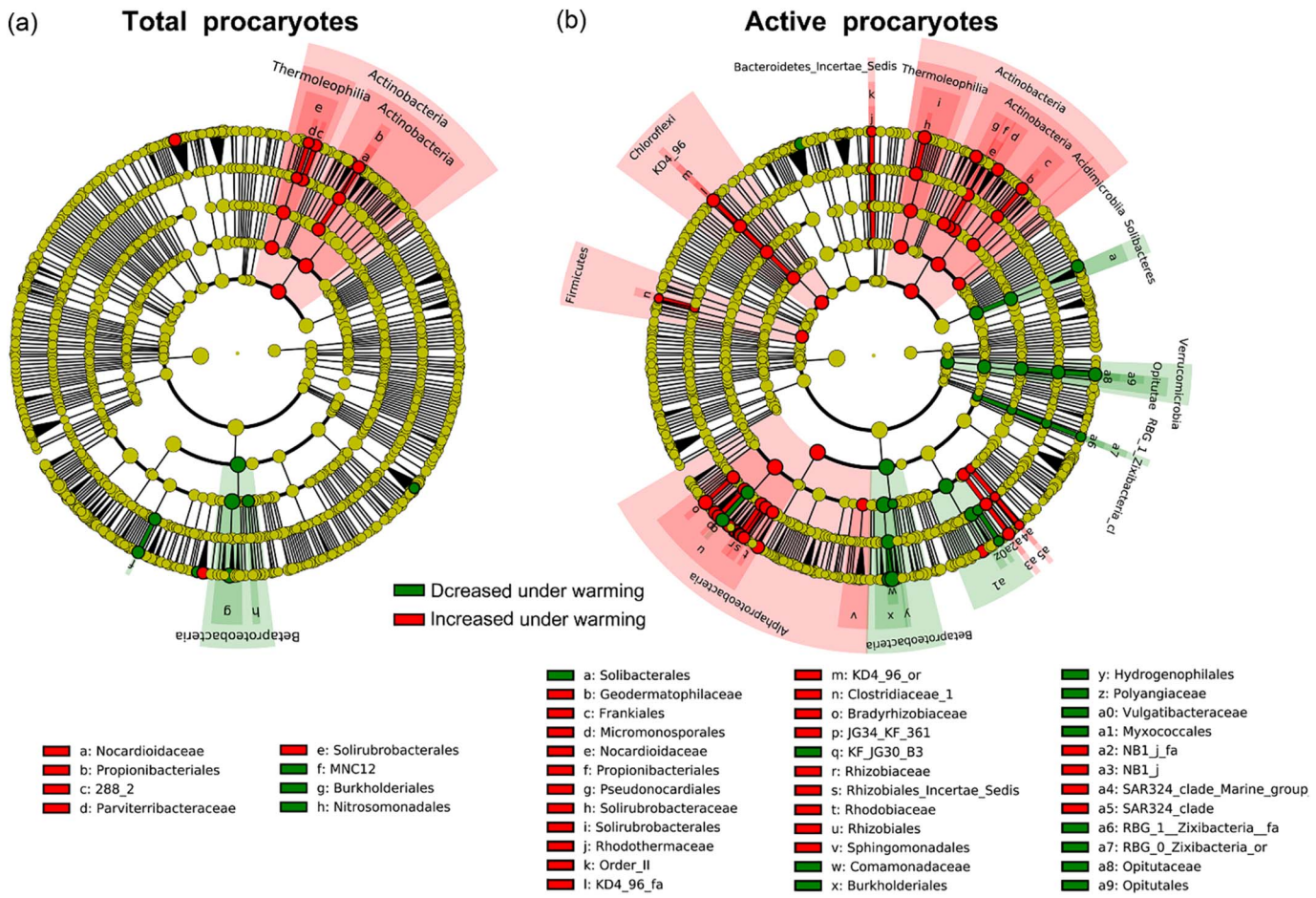

Fig. 5. The responses of the proportions of total (a) and active (b) soil procaryotic lineages to warming. The effects of warming were determined using the LEfSe analysis, and the threshold on the absolute logarithmic LDA score was 3.0.

$P < 0.001$) and active ($r^2 = 0.5381$, $P = 0.007$) procaryotic community structures. The multivariate regression tree analysis showed that the total and active procaryotic community structures were mostly affected by the soil moisture and temperature, respectively (Fig. 7).

3.5. The putative function profiles based on PICRUSt analysis

As shown in Fig. 4c and d, the community structures based on the putative functions showed much higher similarities than the OTU-based community structures among all the samples. The PERMANOVA suggested that the warming significantly altered the active procaryotic community function structures ($P = 0.002$), but the total procaryotic community function structures remained unaffected (Table S2). Again, the grazing and warming-grazing interactions exerted no significant effects on the community function structures (Table S2).

The LEfSe analysis demonstrated that the warming significantly inhibited the fundamental functions such as DNA replication, transcription, translation, protein assembly, signal transduction, cell motility, and secretion (Fig. 8). Some functions related to the degradation of pyrimidine and proteins were also depressed under the warming (Fig. 8). However, the warming also significantly enhanced membrane transportation (e.g., ABC transporters), the metabolism of some substrates (e.g., fatty acids), and the expression of some transcription factors (Fig. 8).

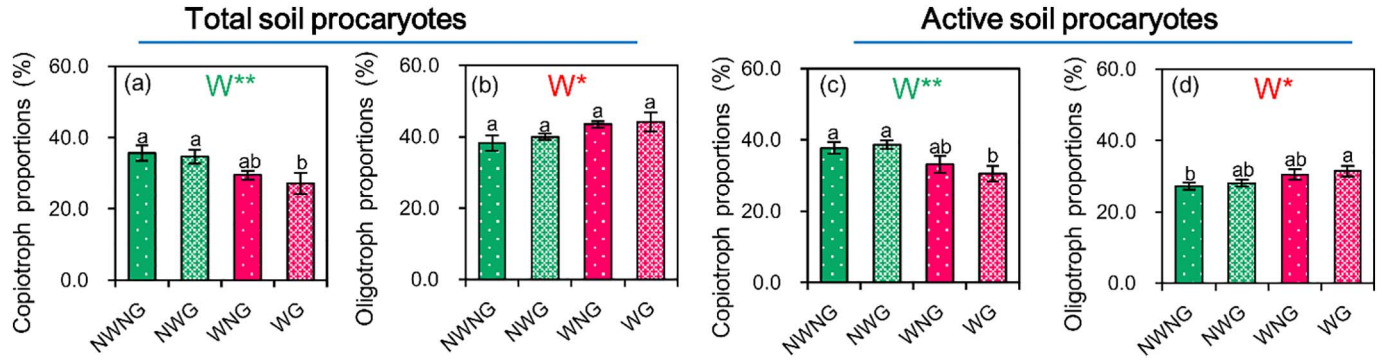

Fig. 6. The relative abundance of copiotrophic and oligotrophic lineages under different treatments. NWNG: no-warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; WG: warming with grazing; W: effect of warming. All the data were presented in mean ± SE, $n = 4$. Bars with different letters indicate significant differences. The copiotrophic lineages included Bacteroidetes, Betaproteobacteria, and Gammaproteobacteria. The oligotrophic lineages included Archaea, Acidobacteria, Actinobacteria, Planctomycetes, Verrucomicrobia, and Chloroflexi. The life-strategy of these lineages was classified based on their responses to organic matter amendments in the published investigations.

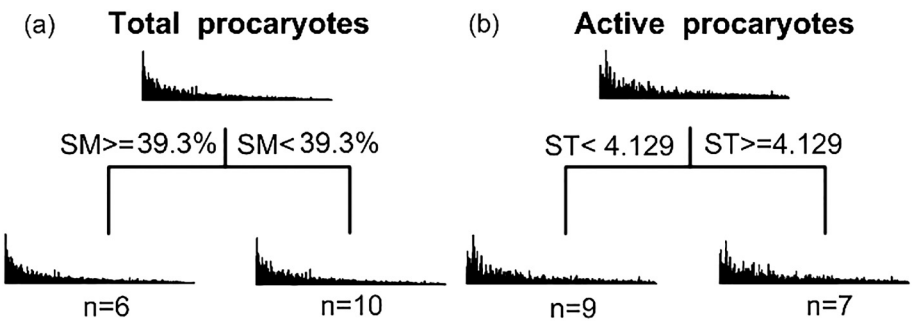

Fig. 7. The relationships between total (a) and active (b) soil procaryotic community structures and the environmental factors, as determined using the multivariate regression tree analysis. SM: soil moisture (%); ST: soil temperature (°C). The bar plots showed the average relative abundances of OTUs in each split groups, and the numbers (n) under the bars represented the sample number within each group.

4. Discussion

Although the previous studies suggested that warming significantly affected the plant communities and soil properties in this alpine meadow (Lin et al., 2011; Luo et al., 2009; Wang et al., 2012), the bacterial and methanotrophic community compositions were highly resistant to three years of warming (Li et al., 2016; Zheng et al., 2012). However, the current study showed that both the total and acitive soil procaryotic community compositions were significantly altered after six years of warming (Table S2, Fig. 4). These findings suggested that the responses of soil microbes to warming could lag behind plant communities and soil properties. Indeed, shifts in microbial community compositions under short-term warming were extensively observed (Xiong et al., 2014; Xue et al., 2016a; Zhang et al., 2016b). Nevertheless, a number of studies showed that the responses of soil microbes to experimental warming were time-dependent (Pold et al., 2016; Romero-Olivares et al., 2017). Sometimes, it took more than one decade to exhibit the first significant response (Rinnan et al., 2009). Moreover, in line with our first hypothesis, compared to the total procaryotic community structures, the active procaryotic community structures showed

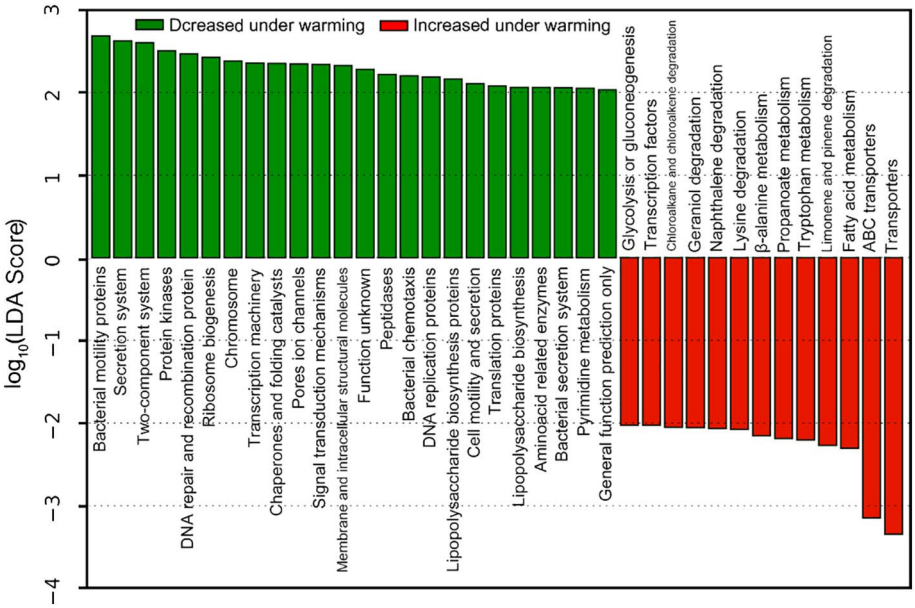

Fig. 8. Responses of the proportions of putative functions to warming. The functional profiles were predicted based on 16S rRNA amplicon sequencing, using PICRUSt analysis with KEGG pathway assignment at level three. The effects of warming were determined using the LEfSe analysis, and only the putative functions with an absolute logarithmic LDA score > 2.0 were shown.

similar but much higher sensitivities to the six-year warming (Table S2, Fig. 4). As proposed by Blazewicz et al. (2013), cellular rRNA concentration represents the growth potential of microbes. Therefore, the higher sensitivity of the active procaryotes should suggest that the responses of the soil procaryotic community to warming could increase with time, which deserves further verification.

Enhanced soil microbial activity under warming has been frequently observed (Lin et al., 2011; Peng et al., 2015; Rinnan et al., 2009; Schindlbacher et al., 2011). However, this study suggested that soil microbes might become less active under the six-year warming compared to no warming, which denied our second hypothesis. First, the warming significantly decreased the relative abundance of β-Proteobacteria, but increased the proportion of Actinobacteria (Fig. 5a). The β-Proteobacteria and Actinobacteria have been respectively classified as copiotrophic and oligotrophic categories (Bernard et al., 2007; Che et al., 2016b; Fierer et al., 2007). Actually, the warming also significantly increased the relative abundance of oligotrophic categories, but decreased the copiotroph proportions (Fig. 6). The shifts in soil procaryotic community composition toward oligotrophic populations indicated a decrease in microbial activity under the warming. This was in agreement with the significantly suppressed soil respiration rates and 16S rDNA transcription under the warming (Fig. 1). Consistently, as revealed by the 16S rRNA-based PICRUSt analysis, the warming also significantly down-regulated the DNA replication, gene expression, and signal transduction (Fig. 8), which provided further evidence for the microbial activity decline under the warming.

In this study, the changes in soil procaryotic communities could be elicited in a number of ways. As revealed by the envfit and multivariate regression tree analysis, the soil temperature and moisture showed the strongest correlations with the total and active procaryotic community structures (Table S3; Figs. 7 and S6). This indicated that the changes in prokaryotic communities might be mainly induced by the soil temperature increase and moisture decrease under the warming (Fig. S1). First, the raised temperature could result in selective microbial growth (Bai et al., 2017; Supramaniam et al., 2016; Xue et al., 2016a), and thus created new warm-adaptive microbial communities which differed from the initial ones. Second, the decreased soil moisture under warming led to decrease in the availability of nutrients (e.g., nitrogen; Fig. S1; Moinet et al., 2016; Xue et al., 2017). This is a possible explanation for the shifts in prokaryotic communities toward oligotrophic populations under the warming (Fig. 6). Third, the increased plant belowground biomass under warming (Fig. S1f) might also elicit higher plant-microbe competition for nitrogen and other nutrients (Classen et al., 2015; Kuzyakov and Xu, 2013), altering the community composition of soil microbes. This is also supported by the significant correlations between the belowground biomass and the relative abundance of copiotrophs and oligotrophs (Fig. S5). In addition, as mentioned above, the responses of soil microbial communities to warming could be time-dependent (Melillo et al., 2017; Metcalfe, 2017; Romero-Olivares et al., 2017). Hence, the past changes in soil and plant properties (Lin et al., 2011; Luo et al., 2010; Luo et al., 2009; Rui et al., 2011; Rui et al., 2012; Wang et al., 2012) might have also contributed to the current alterations in soil microbial community compositions. For instance, the previously observed increases in soil respiration and litter decomposition rates (Lin et al., 2011; Luo et al., 2010) might have resulted in a rapid consumption of labile carbon, and thus decreased the relative abundance of copiotrophic microbial lineages. Collectively, in this study, the changes in procaryotic community structures under warming were probably caused by the combination of both the direct and indirect effects of warming.

The active procaryotic Shannon diversity index, richness, and evenness were all significantly increased under the warming, while the α-diversity of total soil procaryotes remained unaffected (Fig. 2). These findings suggested that more soil procaryotic species were activated by the warming. Additionally, in the Tibetan alpine meadow, the decrease in microbial community dispersions under warming (Figs. 2d and 4a) has been observed and interpreted in the previous studies (Li et al., 2016; Zhang et al., 2016a). Nevertheless, we found that the dispersion of active procaryotic communities remained unaffected by warming (Figs. 2h and 4b). This indicated that the decreased procaryotic community dispersions under the warming might be mainly caused by the responses of the less active procaryotic assemblages. The rRNA to rDNA ratios of oligotrophic lineages were generally lower than those of the copiotrophic lineages (Fig. 3). As the 16S rDNA copies remained unaffected by the warming (Fig. 1a), the significantly suppressed transcription of 16S rDNA (Fig. 1b and c) could be mainly attributed to the decrease in copiotrophs to oligotrophs ratios under the warming.

In consistency with our third hypothesis, the effects of the moderate grazing on soil procaryotes were generally weak, which might suggest that the soil procaryotes had adapted to the treatment due to the long history of grazing at the study site. In addition, grazing mainly affects ecosystem by decreasing litter input (Luo et al., 2009). As fungi are generally copiotrophic and affected more by litter inputs than procaryotes (Che et al., 2016b; Ho et al., 2017; Yarwood et al., 2009), the grazing might have mainly affect the soil fungi rather than procaryotes. Thus, the response of soil fungi, especially the active populations, should be explored in future studies. Nevertheless, in line with our previous studies (Li et al., 2016; Luo et al., 2010; Rui et al., 2012; Wang et al., 2012; Zheng et al., 2012), we detected significant interactive effects between the warming and grazing, which all tended to offset the individual effects of warming and grazing (Figs. 2d, S1c, and S1d). These interactions could be elicited by the contrasting effects of the warming and grazing on soil properties, plant communities, and microbial groups, as interpreted and synthesized in our previous study (Li et al., 2016). This suggested that the moderate grazing could partially counterbalance the effects of global warming on the soil microbes and ecosystem functioning, which further supported our third hypothesis. However, we also found that the interactive effects detected in this study were much weaker than in the previous studies. For example, in contrast to the previous study (Li et al., 2016), the significant interaction effects on the microbial α diversity and community compositions were not detected in this study. This implies that the role of moderate grazing as a counterbalance to the ongoing global warming may diminish over time.

5. Conclusions

This study showed that the six-year warming significantly decreased the 16S rDNA transcription and the microbial respiration rates. It also significantly increased the relative abundances of oligotrophic microbes, and decreased the copiotrophic lineage proportions. The DNA replication, transcription, translation, and signal transduction were significantly depressed under the warming. The grazing significantly decreased soil 16S rDNA transcription and total procaryotic richness, but exerted no significant effects on soil microbial community structures. Collectively, these findings suggest that soil procaryotic community is more sensitive to warming than grazing, and long-term warming can shift the soil prokaryotic community to a more oligotrophic and less active status.

Acknowledgements

We thank Haibei Alpine Meadow Ecosystem Research Station staff for assistance with soil sampling. The research was supported by Strategic Priority Research Program (B) of the Chinese Academy of Sciences (XDB15010201), National Natural Science Foundation of China (41230750 and 41401075), and National Basic Research Program (2013CB956000). Rongxiao Che also received the Joint PhD Scholarship under the agreement between Griffith University and Chinese Academy of Sciences. The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2017.12.005.

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